Biotechnology & its Processes

* Biotechnology deals with → Technique of using Live (organism / enzymes) to produce Organisms from → products / processes useful for humans

in this sense → Making of (curd, bread, wine) which are microbe mediated processes. could also be thought As a form of Biotechnology.

however biotech is used in a restricted sense today. Refer to

Such processes which use → Genetically modified organisms to achieve the same on large scale

Many more processes techniques are also included in biotechnology

for eg:
- "Test tube" baby leading to In vitro fertilization
- synthesising gene
- developing a DNA vaccine
- correcting a defective gene

* EFB (European Federation of Biotechnology) has given definition of Biotechnology that encompasses both traditional view & modern molecular biotech.

definition: Integration of natural science & Organisms, cells, parts thereof, Molecular analogues also enzymes for products & services

definition is as follows

Principles Of Biotechnology

* Among many the 2 core technique enabling birth of biotechnology
  - Genetic Engineering
  - Bioprocess Engineering

Genetic Engineering

Techniques to alter the chemistry of genetic material to introduce DNA / RNA in Host organism. Thus changing phenotype of host.

Bioprocess Engineering

Maintenance of sterile (microbial contamination-free) ambience in chemical engineering processes to enable Growth of only desired microbe / Eukaryotic cells in large quantities for manufacturing Biotech. products (antibiotic, enzyme, vaccine)

* Sexual Reproduction (* Advantage) → provides → variations / formulations → of → Unique combination of Genetic setup → some of which may be beneficial to organisms / population
  - permits variation

* Asexual Reproduction → preserves → Genetic Information

* Traditional Hybridisation procedures → used in → plant breeding / Animal breeding → very often lead to → ① Inclusion ② Multiplication of undesirable genes along with desired ones

* Recombinant DNA / Gene cloning / Gene transfer → includes → Technique of Genetic Engineering → overcome these limitations → and allows us to → isolate / introduce → only 1 set of desirable gene → without introducing undesirable genes into target organism.

* Fate of DNA transferred into a alien organism → most likely → this piece of DNA → not be able to → multiply itself in progeny cells of organism

But → When it gets integrated into genome of recipient → it may → multiply and inherited along with host DNA

This is bcz → Alien piece of DNA → has become → Part of a chromosome → which has → ability to replicate

* On a chromosome → there are → specific DNA sequence → called → Origin of Replication → which is responsible too → initiating replication. → Therefore → Alien DNA linked with this region → can → multiply / replicate → used in → host organism → called as → CLONING

OR → making multiple identical copies of → any template DNA.

* Instance of 1st Recombinant DNA → emerged from → Possibility of linking a gene → encoding → Antibiotic Resistance → with → native PLASMID → of → Salmonella typhimurium

PLASMID:
- autonomously replicating
- circular
- extra-chromosomal DNA

Stanley Cohen & Herbert Boyer accomplished this in 1972 by isolating the antibiotic resistance gene. by cutting out a piece of DNA from Plasmid which was responsible for conferring antibiotic Resistance.

this cutting of DNA at specific locations became possible with discovery of so called MOLECULAR SCISSORS → Restriction Enzymes.

* Cut piece of DNA was linked to Plasmid DNA acts as VECTORS to transfer piece of DNA attached to it

Vector hence plasmid is used as to deliver an alienee piece of DNA into Host organism

* Linking of antibiotic Resistance gene to plasmid vector becomes possible with Enzyme – DNA Ligase which acts on cut DNA molecules and joins their ends This makes New combination of Circular autonomously replicating DNA created In vitro known as Recombinant DNA.

When this DNA was transferred into Escherichia coli (A bact. closely related to Salmonella.) it could Replicate using host's DNA Polymerase and make multiple copies

The ability to multiply copies of antibiotic resistance gene in E. coli was called Cloning of antibiotic resistance gene in E. coli

* 3 basic steps in Genetically Modifying an organism →

1. identification of DNA with desirable genes
2. Introduction of identified DNA into → host
3. maintenance of introduced DNA in host & transfer of it into → progeny

Tools of RECOMBONANT DNA TECHNOL.

Key tools for Genetic Engineering or Recombinant DNA tech
- restriction enzyme
- polymerase enzyme
- Ligase
- vectors
- Host organism

Restriction Enzymes

In 1963 → 2 Enzymes responsible for restricting the growth of bacteriophage in E.coli were isolated.

- Other, cut DNA → RESTRICTION ENDONUCLEASE
- One, added $-CH_3$ grp to DNA

1st Restriction Endonuclease — Hind-II → whose functioning is → depended on a specific DNA nucleotide sequence

Hind-II always cut DNA molecules at particular point ← was found. ← Characterised 5 years later ← was isolated &

at particular point → recognising → Specific sequence of 6 base pairs → this → Specific Base sequence

Recognition Sequence ← is known as

For Hind-II

Besides Hind-II, we know
> 900 — Restriction Enzyme → that have been isolated from
230 strains of bacteria
which recognise → different recognition sequences.

Convention for naming these enzymes is
- First letter comes from → Genus
- Second 2 letters comes from → Species of Prok. cell from which they were isolated.

Example

EcoRI → comes from → Escherichia Coli RY13.
- Roman no. indicate → Order in which enzymes were isolated from that strain of bact.
- derived from → Name of strain

Restriction Enzymes → belong to a larger class of enzymes → NUCLEASES

- Exonucleases → Remove nucleotide from Ends of DNA
- Endonucleases → cuts at specific positions within DNA.

Each Restriction Endonuclease → functions by → "inspecting" the length of DNA sequence → Once it finds specific recog. sequence ← Will bind to the DNA

→ Cut each of the 2 strands of the → Double helix at → Specific points in → sugar phosphate backbones

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* Each Restriction Endonuclease → recognises → Specific palindromic nucleotide sequences in the DNA.

→ These are groups of letters that form same words when read backward & forward — PALLINDROME

→ An DNA sequence ← Base pairs that reads same on 2 strands when orientation of reading is kept the same.

Example → 5' → GAATTC — 3'
3' — CTTAAG ← 5'

* Restriction Enzymes cut the strand of DNA → A little away from the centre of the pallindrome sites.

but → b/w the same 2 bases → on opposite strands

this leaves → Single stranded portion at end.

these are → Overhanging stretches → called → STICKY ENDS (on each strand)

named as → they form H-bond with → their complimentary cut counterparts

* Stickyness of ends facilitate the action of ligase

* Restriction endonucleases → used in → Genetic Engineering → to form → Recombinant molecules of DNA

which are composed of → DNA from different source/genome

* When cut by → Same restriction Enzyme → resultant DNA fragments → have → Same kind of sticky ends.

can be joined by ← DNA ligase

Hence → unless one cuts → vector / source DNA } by → same restriction Enzyme

Recombinant vector cannot be created

Seperation & Isolation Of DNA fragments

⊙ Cutting of DNA → by restriction endonuclease results in → fragments of DNA. → can be separated by technique → Gel Electrophoresis

⊙ DNA fragments → are → -vely charged molecule / DNA can be → Seperated by forcing them to move towards Anode

Medium / Matrix ← through ← Electric field ← under

these days used → Agarose gel (Natural polymer extracted from seeweeds)

DNA fragments seperate (resolve) acc^n to their size only

Smaller the fragment → Farther it moves ← Sieving effects ← through

provided by agarose gel.

Seperated DNA fragments → can be → visualised only after staining with → Ethidium Bromide

followed by → Exposure to UV radiation → DNA fragments can be seen bright orange coloured bands

In Ethidium bromide stained gel exposed to UV light → Separated bands of DNA

Elution

cut out from → Agarose gel → Gel pieces ← extracted from

* DNA fragments → purified this way one → used in → constructing → Recombinant DNA

Cloning vectors ← by joining them with

Cloning Vectors

Plasmids, Bacteriophage → have ability to replicate → within bacterial cells independent of control of chromosomal DNA.

Bacteriophage —(bcz of their)→ high copy no. per cell. —have→ Very high copy numbers within Bacterial cells. ← of their genome

⊙ Some plasmids —may have only→ 1 or 2 copies per cell —whereas others have→ 15-100 copies per cell.

---→ their no's can go even higher

* If we can → Link alien piece of DNA —with→ Bacteriophage / Plasmid DNA —we can→ Multiply its numbers equal to copy no of plasmid / Bacteriophage.

* Vectors used at present → Are engineered in such a way that ① they help in ② easy linking of foreign DNA and ② Selection of recombinants / non recombinants from.

• The following are features that are required to facilitate cloning into a vector :

(i) ORIGIN OF REPLICATION (ORI)

→ Is a sequence where Replication starts

* Any piece of DNA —linked to this→ Sequence —can be made to→ replicate within host cell.

This sequence is responsible for → controlling copy no. of linked DNA

If one wants to recover many copies of the target DNA

↓ It should be

Cloned in a vector having high copy no.

(ii) SELECTABLE MARKER

In addition to → "ori", vector requires a selectable marker.

Selectively permitting the growth of transformants ← non-transformants which helps in ① identifying & ② eliminating

* TRANSFORMATION is a procedure through which a piece of DNA is introduced in → Host Bacterium

* Useful Selectable Marker for E. coli → Genes encoding resistance to antibiotics — ampicillin, chloramphenicol, tetracycline, kanamycine

(iii) Cloning Sites :

In order to link alien DNA → Vector needs to have → Very few, probably single Recognition sites.

Restriction enzyme ← for commonly used

* Presence of more than one recognition sites → within vector will → Generate several fragments → which will complicate the gene cloning

* Ligation of alien DNA is carried out → at a restriction site → present in one of the 2 → Antibiotic Resistance genes

Imp. Example

Ligate a foreign DNA → at → BamH I site of Tetracycline resist. gene (in Vector - pBR322) → Recombinant plasmids (lose the tetracycline Resistance due to insertion of foreign DNA)

Recombinant plasmids (lose the tetracycline Resistance) → BUT STILL → Can be selected out from → non-recombinants

by → plating the transformants on → Tetracycline medium

* Transformants growing on → ampicillin medium → are then transferred to → Tetracycline medium

* RECOMBINANTS → grows in → Ampicillin med. → not grows in → Tetracycline med.

* NON-RECOMBINANTS → grows on → Both antibiotics medium

1 antibiotic resistance gene → helps in → Selecting Transformants

Other antibiotic resistance gene → gets → "inactivated due to insertion" of alien DNA → Selecting RECOMBINANTS ← helps in

* Selection of Recombinants → due to → Inactivation of antibiotics → cumbersome process → bcz

it requires → Simultaneous plating on 2 plates having diff. antibiotics.

Therefore

Alternative selectable marker have been developed

which differentiate

recombinants from non-recombinants.

on the basis of

their ability to produce colour

in the presence of

A chromogenic substance/substrate

In this

A recombinant DNA is inserted within → Coding sequence.

β-Galactosidase ← of enzyme

this results in

Inactivation of the gene for → synthesis of enzyme

referred to as

Insertional inactivation

* Presence of chromogenic substrate gives → Blue coloured colonies

if → Plasmid in Bact. doesn't have insert.

* If insert is present → results in → Insertional inactivation of → β-galactosidase gene

recombinants ← these are

colonies do not produce any colour

(iv) Vectors for Cloning Genes in plants & Animals

Genes can transferred from → Bacteria / Virus to → plants / animals

⊙ How to deliver genes in eukaryotic cells to transform them & force them to do what bact/fungi wants.

Examples →

• Agrobacterium Tumefaciens → A pathogen of several dicot plants

is able to deliver → A piece of DNA known as 'T-DNA' → to transform → A normal plant cell

into → TUMOR → direct those tumor cells to → Produce chemicals required by pathogen

•) Retroviruses → In animals have ability transform normal cell → to cancerous cells

*A better understanding of → Art of delivering genes by pathogen in eukaryotic cell or host has generated knowledge → to transform these tools of pathogen into useful vectors for delivering genes of interest to humans

* Tumor inducing (Ti plasmid) of → Agrobacterium Tumefaciens has now been modified into Cloning vector which is No more pathogenic to plants but still be able to use mechanism to deliver the gene of interest into Variety of plants

* Retroviruses → now been disarmed → are now used to deliver desirable gene into animal cell

* Once a gene / DNA fragment → has been ligated into Suitable vector then transferred to ① Bact. host ② Animal host ③ plant host when it multiplies

COMPETENT HOST for → {Transformation with Recombinant DNA}

DNA → Hydrophilic molecules it cannot → Pass through cell membrane.

In order to force Bacteria to take up DNA → Bact. cells must be made competent to take up DNA

* Treating Bacterial cell with → Specific concentration of divalent cation which → Ca^{2+} eg

DNA enters bacterium through pores in cell wall with which ↑ efficiency

Recombinant DNA can then be forced into such cell. by → incubating them (cells) with Recomb. DNA.

On ice → followed by → placing them briefly at 42°C (heat shock) → then putting back on ice

Bacteria to take up Recombinant DNA ← this enables

* MICROINJECTION → Recombinant DNA directly injected into nucleus of animal cell

Suitable for plants

* BIOLISTICS / GENE GUN → Cells are bombarded with high velocity micro-particles of DNA coated with Gold OR Tungsten

* Using DISARMED PATHOGEN VECTORS → when these are allowed to infect cells they transfer Recombinant DNA into host.

PROCESS OF RECOMBINANT DNA TECHNOLOGY

◉ Steps of Recombinant DNA technology : in sequence

- Isolation of DNA
- fragmentation of DNA by restriction endonuclease
- Isolation of desired DNA fragment
- Ligation of DNA fragment into → vector
- Transferring the recombinant DNA into → host
- Culturing the host cells in a medium at large scale
- Extraction of desired product

1) Isolation Of Genetic Material (DNA) :

Nucleic acid → Genetic material of all → without exception

* In majority of organism it → DNA

* In order to cut DNA by restriction enzymes → it should be in → Pure form [free from other macro molecules]

DNA is enclosed within membrane ← we have to break open cell to release DNA along with macromolecules → RNA, Protein, polysaccharide, lipid

this breaking of cell membr. achieved by → treating → Bacterial cell / Plant tissue / Animal tissue → with enzymes as → lysozyme - Bacteria; cellulase - plant cell; chitinase - fungus

* Genes → located on → Long molecules of DNA → intermixed with → Proteins (as histone)

* RNA → removed by → Ribonuclease treatment

* Proteins → removed by → Protease treatment

* Other molecules → can be removed by → appropriate treatments (Lipases)

* Purified DNA → precipitates out → after addition of chilled ethanol → seen as collection of fine threads in suspension
↳ Seperated by SPOOLING

2) Cutting of DNA at specific Locations:

Restriction Enzyme digestion (R.E) → are performed by → incubating purified DNA molecules with Restriction enzymes

optimal condition ← at (for that specific enzyme)

* Agarose Gel Electrophoresis → employed to check → progression of restriction enzyme digestion

* DNA → being negatively charged → moves toward → anode

(This process is repeated with Vector DNA also)

* Joining of DNA → involves → several process → after having cut the with specific R.E

Source DNA / Vector DNA → from → cut out 'gene of interest'

Source DNA / cut vector → with Space → mixed & ligase added → results in → Formation of Recombin. DNA

3) Amplification Of Gene of Interest using PCR:

PCR - Polymerase Chain Reaction

* In this rkn → Multiple copies of → Gene/DNA of Interest → synthesized → in vitro

(1) Enzyme - DNA polymerase

(2) 2 sets of primers → using

small chem. synthesized → Oligonucleotides → Complementary to the regions of DNA

Enzyme extends primers using → nucleotides provided in reaction.
→ Genomic DNA as template

- If process of Replication of DNA repeated → many times → segment of DNA amplified to ≈ Billion times (1 billion copies)

Such repeated amplification is achieved by use of Thermostable DNA polymerase isolated from Bacteria - Thermus Aquaticus

remains active during high temp. induced denaturation of ds DNA

* Amplified fragment of desired can be used to ligate with vector for further cloning

(4) Insertion Of Recombinant DNA into Host Cell / Organism

Several methods of introducing ligated DNA into recipient cells. These cells after making "competent" to receive & take up DNA (present in its surrounding)

* if Recombinant DNA bearing gene for Resistance to an antibiotic (eg. ampicillin) transfered to E. coli

host cells become transformed into ampicillin-resistant cells. if we spread Transformed cells on Agar plate containing ampicillin → only transformants will grow

untransformed recipients will die. Due to ampicillin resistance gene one is able to Select a transformed cell in presence of ampicillin

- Ampicillin Resistance Gene here called Selectable Marker.

(5) Obtaining the Foreign Gene Product :

When you insert a piece of → Alien DNA → into → cloning vector → then transfer to → Bact. / Plant / Animal cell

alien DNA gets multiplied → In almost all Recombinant technologies → ultimate aim is → Produce a desirable protein

Hence therein → Need for recomb. DNA → to be → expressed → Foreign Gene gets expressed

Expression of Foreign Genes → under → appropriate condition → in host cells → involve → understanding many technical details

After having cloned the gene of interest → having → optimised condition → to induce → Expression of target protein → one has to consider → producing it → on large scale

If protein encoding gene → expressed in → Heterologous host → called → Recombinant protein

Cells harbouring → Cloned gene of interest → may be grown on a → Small scale in laboratory → Cultures may be used for → extracting → desired protein → then → Purifying → by using → Different separation techniques

Cells → can also be → multiplied → in a → Continuous culture system → wherein → used medium → drained out → from one side → while → fresh medium is added from → other → to → maintain the cells → in their → physiologically most active log / exponential phase

this type of culturing method → produces → Larger Biomass → leading to → higher yields of desired protein

small volumes cultures ~~Yield~~ → appreciable quantities of products

• To produce Large quantities → development of Bioreactors

processed ← can be ← culture ← of ← Large volumes (100 - 100 litres) ← where

Thus → Bioreactors are → vessels in which → Raw materials are → Biologically converted

microbial cells / plant cells / animal cells / human cells → using → Specific Products (individual enzymes) etc.. ← into

* A Bioreactors provides optimal conditions for achieving → desired product

Temp. / pH / O_2 / salts / substrate / Vitamins → Optimum Growth Conditions ← by providing

• MOST COMMONLY USED BIOREACTORS — STIRRING TYPE

1) Stirred tank Reactor → cylindrical curved base → to facilitate → mixing of reactor contents

Stirrer facilitates • mixing
• Oxygen availability throughout bioreactor

2) Bioreactor has →
- agitator system
- O_2 delivery system
- Foam control system
- Temp. control system
- pH control system
- Sampling ports → so that small volumes of culture can be withdrawn periodically

Downstream Processing

After completion of Biosynthetic stage → Product

Ready for marketing ← before it is ← series of processes ← subjected through

as → product.

* Process includes → separation, purification → collectively referred to as → Downstream processing

Product → has to be formulated with → suitable preservatives

Such formulations → has to undergo through → clinical trials → as in case of → Drugs

Requirement – Strict Quality Control testing → (for each product) required

Downstreaming processing
Quality control Testing
→ vary from product to product

Date / /

* Genetic Engineering is possible bcz – we can cut DNA at → specific sites by Restriction Endo nuclease.

- KEY FACTOR → which make → plasmid vector in genetic engineering
  - → its ability to carry a foreign gene.
- Sticky ends are → unpaired base pairs
* See diagram on Pg no. 196 [EcoRI cuts that GAATTC]
  Pg no. 147
* Remember the steps of Recombinant DNA technology in order.
* Transposons are → Jumping genes
- Taq polymerase
  - → Remains active during high temp induced denaturation of dsDNA
  - → Requires primer for carrying out process of polymerisation
  - → synthesises RNA region b/w primers
  - ✗ using dNTPs & Mg^{2+} — wrong statement
- Denaturation – 94°C
  Annealing – 56°C
  Extension – 72°C.
* Kary Mullis – PCR
* Paul Berg – Father of Genetic engineering
* Arber, Nathan, Smith – Nobel prize for discovery of Restriction Endonuclease
* Vectors → Plasmid
  - → Cosmid
  - → Phagemid
* Agarose – Polymer.
* pBR322 – Artificial Vector. (Plasmid; Bolivar → Rodriguez.)
  - → number that distinguish it from other plasmid.
* Vectorless Transfer Methods
  - → Microinjection
  - → Biolistics
  - → Gene gun
* Features of plasmids
  - → Transferable
  - → Independent replication
  - → Circular str.
* Total no. of restriction sites in pBR322 — 8
* Have recognition sequence of 6 base pairs → EcoRI, Hind II
* 20th century offshoot of modern biology is – Biotechnology

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Boyer performed in 1969, studies on a couple of Restriction Enzyme, of E. coli.

* Palindromic sequence – when read on 2 strands in same polarity.

* Artificial recombinant DNA was constructed for 1st time using
  - A gene encoding antibiotic resistance.
  - Extrachromosomal DNA of Salmonella typhimurium

* tpA → clot busts / used in myocardial infection
  Intaferon - α → used in treatment of Hepatitis B

* Enzymes – smaller
  DNA – bigger
  (cz enzymes are proteins synthesised from DNA)

Q. What would be molar conc. of human DNA in human cell?

Molar conc = N_A X Total no. of chromosome
= $6.023 \times 10^{23} \times 46$
= 2.77×10^{23} moles

* Eukaryotic cells – don't have Restriction Endonuclease

* Phase of meiosis where Recombinant DNA formed – Pachytene

* Reporter Enzyme – selectable marker